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FUNCTIONAL CHARACTERIZATION OF *ANOPHELES GAMBIAE* SPÄTZLE GENE FAMILY

by
HIBA MOHAMAD ZEIN EL DINE

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HIBA MOHAMAD ZEIN EL DINE

Approved by:

Dr. Mike Osta, Professor
Department of Biology — Advisor

Dr. Sawsan Kuraydiyyah, Professor
Department of Biology — Member of Committee

Dr. Zakaria Kambris, Associate Professor
Department of Biology — Member of Committee

Date of thesis defense: July 18, 2022

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Student Name: Zein El Dine	Hiba	Mohamad
Last	First	Middle

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ABSTRACT
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Mosquito vectors of diseases are not passive hosts for the pathogens they transmit, rather they employ a robust innate cellular and humoral immune responses against the various microbes they encounter. In *Drosophila* and other insect species, a key immune signaling pathway, the Toll pathway, provides resistance against fungal and bacterial infections, mainly Gram-positive bacteria, and is a major regulator of the expression of several immunity genes, specifically those encoding antimicrobial peptides (AMPs), as well as other genes involved in the stress response. In insects, Toll is activated by binding to a cleaved active form of the cytokine molecule Spätzle (Spz). Spz cleavage is regulated by a cascade of clip-domain serine proteases (CLIPs). Despite being well characterized in *Drosophila*, our knowledge of the Toll pathway activation remains largely fragmented in the major African malaria vector *Anopheles gambiae*, and mosquitoes in general, specifically that several components of this pathway have not been characterized yet.

Here, we functionally characterize by RNA interference (RNAi) the roles of the six Spz genes (Spz1-6) identified in the *A. gambiae* genome in immune responses to Gram-positive and Gram-negative bacterial and fungal systemic infections. In the context of mosquito tolerance to *Staphylococcus aureus* infections, silencing of Spz genes resulted in mixed phenotypes, with *Spz2* and *Spz4* knockdown (kd) compromising survival in more trials compared to other candidates. Similarly, *Spz2* kd significantly compromised mosquito survival to fungal infections in most of the conducted trials, while the other Spz genes gave variable phenotypes, suggesting that Spz2 may be indispensable for mosquito tolerance to fungal infections, in addition to its less prominent role against Gram-positive bacterial infections. However, none of the Spz gene kd compromised mosquito survival to *Serratia marcescens* systemic infections, suggesting that the Toll pathway may not be a key player in the immune defense against Gram-negative bacteria in *A. gambiae*. Spz gene silencing did not alter mosquito resistance to bacterial and fungal infections, indicating that the Toll pathway may not play a major role in bacterial and fungal clearance in the mosquito, as it does in *Drosophila*. QRT-PCR analysis revealed that most of the Spz genes were upregulated after fungal infections, and particularly peaked at 48 hours after infection, whereas none were induced after *S. aureus* challenge, revealing that the Toll pathway may be more implicated in anti-fungal defenses. A better understanding of the activation of the mosquito Toll pathway, its relevance to host defense, and the functional characterization of its components contributes significantly to our knowledge on mosquito-pathogen interactions, which would be especially relevant in the context of fungal infections since entomopathogenic fungi are being considered as potential biopesticides for the control of mosquito vectors

of disease. Hence, a better characterization of this pathway would help understand the potential mechanisms that may be used by fungi to evade mosquito immune responses.

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INTRODUCTION

A. *Anopheles gambiae*: A Primary Vector of Malaria

Vector-borne diseases, particularly malaria, remain a global threat to public health. In 2020, there were an estimated 241 million malaria cases and 627 000 malaria deaths according to the latest World Health Organization report [1], indicating that malaria control reached a plateau in the last three years. The African Region continues to account for the highest share of the global malaria burden, being home to 95% of all malaria cases [1].

Malaria is caused by *Plasmodium* parasites which include six species able to infect humans: *P. falciparum, P. vivax, P. ovale, P. malariae*, *P. knowlesi,* and *P. cynomolgi* [2], with the first two species posing the greatest threat. The parasites are transmitted to human hosts by female infected mosquitoes of the genus *Anopheles* [3]. There are around 530 recognized species of *Anopheles* [4], however, only 30-40 are found to transmit human malaria in nature [5]. *Anopheles gambiae* is the best studied, being the major malaria vector in Africa with a prominent role in the transmission of *P. falciparum. A. gambiae* sensu stricto is part of a complex of eight morphologically indistinguishable sibling species which differ in their geographical and ecological distribution. As a result of speciation, *A. gambiae* s.s. is split into two molecular forms – the Mopti (M), now referred to as *A. coluzzii* [6], and Savanna (S) forms – which display pronounced genetic differentiation [7]. Although mosquitoes can be seen as passive hosts, they can employ multiple innate immune responses against the various microbial challenges they encounter.

B. Mosquito Innate Immune Responses

Unlike mammals, mosquitoes lack an adaptive immune system and are dependent on innate immunity to fight infections. When pathogens cross the mosquito's physical barriers and gain entry into the insect, the insect mounts a vigorous cellular and humoral immune response to target the invading pathogen. The best characterized mosquito cellular defenses are those mediated by hemocytes and gut epithelial defenses [8]. The hallmark of the humoral reactions is the production of antimicrobial peptides (AMPs) by the fat body and their secretion in the hemolymph upon microbial infection [8-10]. Other humoral responses include melanization [11], and complement-like complex [12].

1. Mosquito Cellular Defense

a. Hemocyte-mediated Defense

The hemolymph of *A. gambiae* mosquito contains three types of immune effector cells or hemocytes: granulocytes, oenocytoids, and prohemocytes which are morphologically and functionally distinct. Granulocytes are the most abundant cell type, while oenocytoids and prohemocytes constitute less than 10% of the total hemocyte population [13]. Granulocytes are polymorphic and contain numerous membrane-delimited vesicles in the cytoplasm [14, 15]. They are highly phagocytic and are distinguished by their strong acid phosphatase activity [16]. Oenocytoids, on the contrary, are non-phagocytic and are distinguished by their ability to produce phenoloxidase (PO) [13-15], which is the rate-limiting enzyme in the melanization response. The role of prohemocytes remains unclear, as it has been hypothesized to serve as hematopoietic progenitors of the two other cell types [13], however, a study

showed that these cells are phagocytic and may be produced from the asymmetric division of granulocytes [17]. Recently, single-cell studies demonstrated an increased complexity of hemocyte populations beyond these defined cell subtype classifications [18, 19]. Transcriptional profiles and molecular markers identified previously unknown granulocytes subpopulations such as megacytes which are defined by a unique transmembrane protein marker and a high expression of LPS-Induced TNF-alpha transcription factor 3 (LL3), in addition to antimicrobial granulocytes that expressed characteristic AMPs, and proliferating granulocytes [19].

In terms of their distribution, approximately 75% of the total hemocytes in adult mosquitoes are in circulation while 25% are sessile [17]. The majority of sessile hemocytes are present on the abdominal wall, mostly at the periostial regions surrounding the valves of the heart, which is a strategic location for microbial capture since these areas are subject to high hemolymph flow [17, 20]. In addition, a significant number of sessile hemocytes occur in the thorax, head, and appendages. Although the approximate number of circulating hemocytes continues to be debated [21-24], it is known that this number decreases with age and increases in response to infection [17].

The most important immune effector function of hemocytes is phagocytosis, which is an evolutionarily conserved process based on the recognition, engulfment, and intracellular destruction of bacteria and other small foreign entities. In this reaction, the foreign body, recognized by pattern recognition receptors (PRRs) found on the surface of hemocytes or by soluble PRRs functioning as opsonins, is internalized into a phagosome which then fuses with a lysosome causing the degradation of the pathogen [25]. Mosquito granulocytes are highly phagocytic and can initiate this response soon after pathogen exposure [14, 17, 26], with the ability to phagocytose hundreds of

foreign particles within 24 hours of infection [27, 28]. The phagocytic response of mosquito hemocytes is shown to be effective against Gram-positive and Gram-negative bacteria, as well as yeast, *Plasmodium,* and small inanimate particles [14, 26, 27, 29-31]. However, phagocytosis is unlikely to be effective against *Plasmodium* parasites since only 2% of *A. gambiae* hemocytes were shown to engulf *Plasmodium berghei* sporozoites, indicating that this immune response alone does not explain the massive losses of sporozoites that occur in the hemolymph during their migration to the salivary glands [27].

Several mosquito phagocytic receptors have been described including, a β integrin BINT2, a peptidoglycan recognition protein PGRP-LC, and a low-density lipoprotein receptor-related protein LRP1 [32, 33]. Whether these receptors recognize pathogens directly or after the pathogens have been opsonized remains elusive. The most studied opsonin is the complement-like protein, thioester-containing protein 1 TEP1, which following proteolytic activation, tags foreign pathogens for elimination by phagocytes [34]. Other putative PRRs exhibiting roles in phagocytosis include TEP3, TEP4, leucine-rich repeat-containing proteins (LRRs) LRIM1, fibrinogen-related proteins FBN8, and DSCAM which is a hypervariable Ig-containing receptor [32, 33, 35].

In addition to their significant role in phagocytosis, hemocytes can also produce soluble immune factors that contribute to humoral effector responses, indicating that an effective crosstalk exists between cellular and humoral immunity. In fact, mosquito hemocytes produce several factors involved in the melanization response such as multiple phenoloxidases, dopachrome conversion enzyme, clip-domain serine proteases (CLIPs), serine protease inhibitors (SRPNs), and C-type lectins [18, 19, 36-38].

Mosquito hemocytes are also involved in synthesizing cytotoxic effector molecules following an immune challenge. In addition to the hemocyte-produced Tep1 that targets *Plasmodium* parasites, bacteria [39], and fungi [40] for killing, AMPs, reactive oxygen, and nitrogen intermediates are also produced by hemocytes [36, 38].

b. Gut Epithelial Immunity

To successfully establish an infection, *Plasmodium* ookinetes need to invade and traverse the midgut epithelial cell wall to transition into oocysts at the basal side of the epithelium. This midgut traversal by ookinetes is considered a very critical step of the *Plasmodium* lifecycle as the parasite needs to escape from the invaded epithelial cells unharmed. In fact, midgut invasion causes extensive damage to epithelial cells leading to apoptosis. As a response, the invaded cells mount a strong nitration reaction creating a toxic intracellular environment for the traversing ookinetes and marking them for immune recognition by the mosquito complement-like protein TEP1 [41-43]. The epithelial nitration response requires the function of the JNK pathway which seems to induce the expression of the enzymes heme-peroxidase 2 (HPX2) and NADPH oxidase 5 (NOX5) which potentiate nitration in response to *Plasmodium* invasion of the midgut. As the invaded cell is undergoing apoptosis and is being expelled towards the midgut lumen, adjacent cells extend lamellipodia to cover up the damaged area and seal the generated wound [44]. The invaded cell itself is also shown to extend a similar lamellipodia structure which tightly covers the ookinetes like a ‘hood’ during their egress from the epithelium [44]. Microscopic observations revealed that this actin-rich structure surrounds 60% of ookinetes exiting the epithelium, whereas in refractory strains most dead parasites were surrounded by an actin hood [45]. Silencing WASP, a

positive regulator of actin polymerization, causes a significant reduction in hood formation as well as allows the development of some oocysts in refractory strains [45], supporting the hypothesis that the hood is indeed an epithelial cellular defense response against parasites. A study also found that, in refractory mosquitoes, the formation of the actin zone is strongly linked to the activation of the melanization response [46]. Two factors were identified to be involved in controlling both mechanisms: the transmembrane receptor frizzled-2 (Fz2) and the guanosine triphosphate-binding protein cell division cycle 42 (Cdc42) [46]. Silencing of either gene disrupted the assembly of the actin zone as well as melanization but did not lead to a significant increase in the numbers of live oocysts in refractory mosquitoes, indicating that these generally thought killing mechanisms may rather represent a form of mosquito wound-healing response that is triggered by the invading parasite.

Ookinete invasion of the mosquito midgut has been shown to trigger a strong hemocyte priming effect by bringing the gut microbiota into direct contact with midgut epithelial cells [23]. The observed priming effect was later related to the secretion of a hemocyte differentiation factor (HDF), an Evokin/Lipoxin complex, into the hemolymph by gut epithelia, which increases the proportion of circulating hemocytes and induces priming when transferred to naïve mosquitoes [47]. Midgut epithelial cells were shown to produce and release prostaglandin E2 (PGE2), which attracts hemocytes to the midgut basal surface, triggering HDF production and establishing a long-lasting cellular immune response to *Plasmodium* infection [48].

2. Mosquito Humoral Defense

a. Antimicrobial Peptides (AMPs)

Antimicrobial peptides (AMPs) are small and cationic immunity proteins that can act against a broad spectrum of microorganisms. They are produced in response to infection by the fat body and hemocytes from where they are released systematically into the hemolymph [49] or secreted locally by epithelia [50]. The positively charged amphipathic feature of AMPs facilitates their interaction with the negatively charged pathogen surface, resulting in membrane permeabilization, cell lysis, and eventually death [51]. AMPs can also enter cells without membrane disruption, interfere with cellular metabolism, target cytoplasmic components, and inhibit essential intracellular functions [52]. Insect AMPs are classified into four groups based on their structures and amino acid sequence: the α-helical peptides (e.g., cecropin and moricin), cysteine-rich peptides (e.g., defensin, drosomycin, and gambicin), proline-rich peptides (e.g., apidaecin, drosocin, and lebocin), and glycine-rich proteins (e.g., attacin and gloverin) [53, 54]. In *Drosophila melanogaster*, seven well-known classes of AMPs have been identified: defensin, cecropin, attacin, diptericin, drosocin, drosomycin, and metchnikowin, and the regulation of these AMPs genes by the Toll, Imd, and JAK/STAT pathways have been well studied [55, 56]. More recently, novel AMP genes were identified in *Drosophila* which include Bomanins [57], and Baramicin A [58]. Bomanins are a family of 12 AMP genes that play important roles in defense against Gram-positive bacteria and fungi, whereas Baramicin A is cleaved into smaller peptides providing protection against fungal infections.

Defensins (DEF) are particularly active against Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus,* and *Bacillus megaterium*.

Nevertheless, a small number of insect defensins were shown to act against Gram-negative bacteria such as *Escherichia coli* [59, 60] as well as filamentous fungi and yeast strains [61]. Defensins bind to the microbe cell membrane or form pores or channels allowing the efflux of essential nutrients and ions [62]. The first identified AMP was isolated from the hemolymph of the lepidopteran *Hyalophora cecropia* and was named cecropin. Cecropins (CEC) were later shown to be mainly active against Gram-negative bacteria and, to a lesser extent, against Gram-positive bacteria [63, 64], as well as fungi [65]. They act by lysing bacterial cellular membranes, causing leaky membranes, and inhibiting proline uptake [64]. Attacins are mostly active against Gram-negative bacteria, particularly *E. coli* [66], and function by increasing the permeability of the bacterial outer membrane [67] as well as by inhibiting the synthesis of bacterial outer-membrane proteins even without entering the cytoplasm [68]. Similarly, diptericin and drosocin are mostly effective against a limited range of Gram-negative bacteria [62], whereas drosomycin and metchnikowin exhibit potent antifungal activity [69, 70]. Metchnikowin was also shown to have antimicrobial activity against Gram-positive bacteria, but not Gram-negative bacteria.

The *A. gambiae* genome encodes four classes of AMPs, three of which are shared with *Drosophila*: defensin, cecropin, and attacin, while the fourth is mosquito-specific and is known as Gambicin (GAM) [71]. In fact, four DEF and four CEC exist in *A. gambiae*, in addition to one GAM and one yet uncharacterized attacin [71]. The first DEF isolated in *A. gambiae*, DEF1, was found to be constitutively expressed in the midgut and upregulated in the hemolymph following immune challenges [72]. It was active *in vitro* against most Gram-positive bacteria and some species of filamentous fungi but did not show inhibitory effects on yeast or Gram-negative bacteria, except for

few *E. coli* strains [72]. DEF1 knockdown (kd) by RNAi compromised mosquito survival to Gram-positive bacterial infections, however, it did not show any effect on the development and morphology of the different stages of *P. berghei*, indicating that this peptide may not act as an anti-parasitic factor in *A. gambiae* [73]. As for DEFs 2, 3, and 4, they exhibited low-level constitutive expression during all life stages, even following immune challenge, but showed significant increases in mRNA abundance during the larval stages [74]. Conversely, cecropin displayed activity against a broader range of microorganisms: its expression was induced following infection with bacteria and *Plasmodium* and it was shown to act against Gram-positive and Gram-negative bacteria, filamentous fungi, and yeast [75]. Like other *A. gambiae* AMPs, gambicin is induced upon infection in the midgut, fat body, and hemocytes. It was among the first anti-*Plasmodium* factors identified. In fact, gambicin expression is induced during early and late stages of malaria infection. *In vitro* experiments showed that the mature gambicin peptide is active against Gram-positive and Gram-negative bacteria, has a morphogenic effect on filamentous fungi and is marginally lethal to *P. berghei* ookinetes [76]. Both Toll and Imd pathways were shown to regulate the expression of DEF1, CEC1, and GAM1 [77].

b. Melanization

Melanization is an immune effector response that is triggered locally in response to cuticle injury or systemically following microbial invasion of the hemocoel [11, 78, 79]. It is characterized by the synthesis of the dark brown pigment melanin and its cross-linking with molecules on microbial surfaces or in injured areas resulting in the killing of the invader and hardening of the wound clot. In addition to its role in

immunity, melanization is essential for egg chorion tanning and cuticle sclerotization which leads to the hardening of the exoskeleton by cross-linking the cuticular proteins by quinones generated during that process [80].

Melanization requires the activation of prophenoloxidase (proPO) to its active form phenoloxidase (PO), a key enzyme that mediates the oxidation of tyrosine to dihydroxyphenylalanine and the oxidation of dihydroxyphenylalanine and dopamine to their respective quinones which are precursors of melanin formation [81]. The conversion of the proPO zymogen into active PO is achieved by a clip-domain serine protease (CLIP). CLIPs contain one or more amino-terminal clip domains connected by a linker sequence to a carboxyl-terminal serine protease domain and are present in the hemolymph of insects and other arthropods [78]. Histidine, Aspartate, and Serine amino acid residues located in the active site of the protease domain contribute to the acyl transfer mechanism of catalysis of proteolytic CLIP proteases, hence referred to as clip-domain containing serine protease (cSPs). However, not all CLIPs are catalytic; those lacking one or more of the three amino acids residues are proteolytically inactive and are referred to as clip-domain containing serine proteinase homologs (cSPHs). Phylogenetic analysis of mosquito CLIPs based on whole sequence alignment led to their classification into five groups (A to E), whereby CLIPAs are non-catalytic, CLIPBs, CLIPCs, and CLIPDs are mainly active cSPs, while CLIPEs are either non-catalytic or mixed cSP-cSPHs (i.e. containing both catalytic and non-catalytic domain) [37, 71, 82]. CLIPs act in cascades to modulate several immune responses including coagulation, melanization, and synthesis of AMPs through Toll pathway activation. The general scenario of activation of CLIP cascades according to studies from diverse insect species involves first the recognition of microbial surface molecules by PRRs which

interact with and lead to the auto-activation of an upstream modular serine protease (ModSp) which in turn activates a CLIPC that activates a downstream CLIPB [11]. CLIPB then cleaves and activates an effector molecule, such as proSpätzle to form an active Toll ligand leading to the synthesis of AMPs, or proPO which is required for the melanization response. The activity of CLIPs is tightly regulated by serine protease inhibitors known as serpins (SRPNs), which, upon cleavage, form a covalent complex with their target protease, leading to its elimination from the hemolymph [83, 84].

RNA interference (RNAi) based functional analysis in *A. gambiae* identified initially CLIPA8 as an essential positive regulator of the melanization response to *P. berghei* parasites [85], bacteria [86] as well as the entomopathogenic fungus *Beauveria bassiana* [40]. CLIPA8 activation cleavage is controlled by TEP1 and its positive regulator SPCLIP1, suggesting that they act upstream of CLIPA8 in the melanization response [87]. More recently, CLIPA28 was identified as another essential positive regulator of the infection-induced melanization response whereby it functions downstream of CLIPA8 [88]. CLIPB3, B4, B8, and B17 also contribute to the melanization of *P. berghei* ookinetes to different extents whereas CLIPA2, and A14 are negative regulators of this response [85, 89, 90]. This response in *A. gambiae* is tightly controlled by SRPN2; silencing of *SRPN2* increased the number of melanized *Plasmodium* ookinetes and reduced their ability to invade the midgut epithelium, suggesting that it may be regulating different CLIPs involved in separate melanization pathways [91].

In addition to CLIP cascades, a complex of two C-type lectins, CTL4 and CTLMA2 (henceforth CTL complex) act as a key negative regulator of the mosquito melanization response to *Plasmodium* parasites [92], bacteria [93], and fungi [94].

Silencing CTL4 by RNAi triggers the melanization of the majority of *P. berghei* ookinetes invading the mosquito midgut epithelium, whereas the knockout of CTL4 by CRISPR/cas9 leads to the complete melanization of *P. berghei* ookinetes and a strong but partial melanotic response against the human malaria parasite *P. falciparum* [94], indicating that the CTL complex is a key regulator of the melanization response to malaria parasites.

c. Complement-like response

The complement-like system in *A. gambiae* has emerged as a key anti-plasmodial defense mechanism. The hallmark protein in this system is TEP1, homolog to the mammalian C3 complement factor [34], which binds to the surface of ookinetes soon after they cross the midgut epithelium and causes their death by an unknown mechanism that presumably involved parasite lysis [95]. Ookinete lysis in the basal labyrinth of the midgut epithelium seems to account for the major parasite losses associated with midgut invasion [95].

TEP1 is constitutively produced by hemocytes and is secreted into the hemolymph as a full-length protein (TEP1-F) which is constitutively cleaved by a yet unidentified protease. The processing generates two cleaved parts of TEP1 which remain connected by non-covalent interactions [96]. The mature and active form TEP1cut then circulates in the hemolymph and binds to the surfaces of bacteria and parasites through a thioester bond, marking them for killing [34]. TEP1cut is instantly stabilized in circulation by a disulfide-linked heterodimer complex of two LRR proteins, LRIM1 and APL1C [97, 98]. LRIM1 and APL1C not only stabilize circulating TEP1 but also stabilize each other prior to their interaction with TEP1 and this complex

is shown to be required for TEP1 localization and accumulation on the surface of midgut-invading ookinete. Knocking down any of these LRR genes results in the depletion of TEP1cut from the hemolymph by deposition on self-tissues instead of pathogen tissues [98], revealing that the TEP1cut/LRIM1/APL1C complex functions as a complement-like system for parasite killing.

In addition to controlling the lytic response to *Plasmodium* parasites, TEP1 is so far the most upstream positive regulator of the mosquito infection-induced melanization response playing a key role in controlling the activation cleavage of key downstream CLIPs [87, 88]. TEP1 is also involved in the removal of damaged sperm cells in males during spermatogenesis, a function that requires also LRIM1 and the nitration of damaged cells by the enzyme Hemeperoxidase 2 [99].

C. Toll Immune Signaling Pathway

Insects are constantly threatened to be invaded by different kinds of pathogens. As a result, they have developed powerful mechanisms to counter the invading microorganisms. These mechanisms involve the recognition of the infectious non-self and its discrimination from self-tissues as well as effector systems that efficiently target the invader. The detection of microorganisms depends on germline-encoded receptors that recognize repeated patterns of molecular structures that are found on the surface of microorganisms and absent from eukaryotic cells. Following the recognition, several genes including AMPs are induced to target the invading microbial. In insects, three signaling pathways are involved in regulating the expression of AMPs in the fat body; Toll, immune deficiency (Imd), and JAK-STAT pathways which are best characterized in *D. melanogaster* [9]. In *Drosophila*, the Toll pathway is primarily elicited by Gram-

positive bacteria and fungi [100] whereas the Imd pathway is activated by Gram-negative bacteria [101]. As for the JAK-STAT pathway, it has been implicated in antibacterial and antiviral defense in *Drosophila* [102].

1. Drosophila *Toll Pathway*

a. Pathogen Recognition in the Toll Pathway

The initiation of Toll signaling pathway requires a specific interaction between the host's PRRs acting upstream of Toll and pathogen-associated microbial patterns (PAMPs). Several classes of PRRs exist however only peptidoglycan recognition proteins (PGRPs) and Gram-negative bacteria binding proteins (GNBPs) are shown to be involved in the activation of the Toll pathway.

PGRPs form a large group of proteins, conserved from insects to mammals, which recognize and bind to peptidoglycan, an essential and unique bacterial cell-wall polymer [103]. They all share a 160-amino acid domain (the PGRP domain) with notable sequence similarity to bacterial amidases which cleave peptidoglycans [104, 105]. In some PGRPs, the amidase function is conserved, whereas, in others, the enzymatic activity is lost due to a cysteine residue replacement in the PGRP domain [106]. PGRPs are classified into short and long forms. The small-sized PGRPs (PGRP-S) are thought to be extracellular proteins secreted in different parts of the body, whereas the large-sized PGRPs (PGRP-L) are either intracellular or membrane-associated [107]. The *Drosophila* genome encodes 13 genes belonging to the PGRP family, seven of which are small and six are long forms [107]. In *Drosophila*, PGRPs are expressed in immune-responsive tissues including the fat body, hemocytes, the gut, and the epidermis [107].

GNBPs belong to the β-1, 3-glucanase protein family with an amino-terminal β-1,3-glucan-binding domain and a carboxyl-terminal region similar to the β-1,3-glucanase domain [108]. GNBPs were first identified from the hemolymph of the silkworm *Bombyx mori* having a strong affinity to Gram-negative bacteria [109], however, in *Drosophila*, GNBPs are not involved in the recognition of Gram-negative bacteria. The *Drosophila* genome encodes three GNBPs: GNBP1 is expressed throughout *Drosophila* life cycle whereas GNBP2 and GNBP3 showed weak expression during embryogenesis [110].

Toll pathway activation by Gram-positive bacteria requires the function of a complex which includes PGRP-SA and GNBP1 [111-113]. In fact, upon recognition of Gram-positive bacteria, and precisely lysine-type peptidoglycan (PGN) (Lys-type PGN), PGRP-SA and GNBP1 physically interact and form a complex. GNBP1 then seems to hydrolyze Lys-type PGN and present it in a processed form to PGRP-SA [114]. GNBP3 also leads to the activation of the Toll pathway in response to fungal infection by binding to β-1,3-glucan of the fungal cell wall [115]. A modular serine protease (ModSp) relays the signal downstream of GNBP3 and the PGRP-SA/GNBP1 complex to the serine protease cascade that culminates in the cleavage of the cytokine-like molecule Spätzle [116].

b. Serine Protease Cascade Activates Toll Pathway

Upon Gram-positive bacterial PGN binding to the PGRP-SA/GNBP1 complex or fungal glucans binding to GNBP3, an extracellular protease cascade is initiated and terminates by the activation of the Toll receptor. First, following microbial sensing, a modular serine protease, ModSp, is autoactivated upstream of Toll [116]. Using a

ModSp-GFP fusion protein, it was found to be secreted from the fat body and localized at the surface of lipid vesicles circulating in the hemolymph [116]. ModSp does not contain a CLIP-domain but activates a downstream serine protease cascade, which includes Grass, Spirit, Sphinx1/2, and Spheroid, and terminates by the activation of the spätzle-processing enzyme (SPE) [117]. Grass was first identified to be associated with the detection of Gram-positive bacteria [117], hence the name Gram-positive specific serine protease, but it was later shown to be also associated with the recognition of fungal infections [118]. Downstream of Grass, the CLIPs Hayan and Persephone function redundantly to regulate Toll pathway activation [119]. However, it remains unresolved whether Hayan and Persephone act upstream or downstream of Spirit. Spheroid and Sphinx1/2 do not possess a proteolytic activity since their protease-like domain lacks the catalytic cysteine residue, revealing that they may have regulatory functions. In fact, silencing of Spheroid and Sphinx1/2 induced the same phenotype as Spirit and SPE, suggesting that they may act as adaptors or regulators of these two downstream proteases, possibly by localizing them close to their effector molecules [117]. SPE is the terminal CLIP protease of the cascade that leads to the activation of the Toll ligand Spätzle (Spz) [120]. SPE is a zygotic gene constitutively expressed in immune tissues and upregulated in response to infection with Gram-positive bacteria and fungi [121]. SPE acts upstream of Toll and is required specifically for the cleavage of Spz, and the induction of SPE transcription is controlled by the Toll pathway, thereby generating a positive feedback loop [121].

SPE can also be activated independently of PRRs through a protease cascade mediated by Persephone (Psh). Psh, a CLIP-domain containing serine protease, is proteolytically matured by secreted fungal virulence factors such as the fungal protease

PR1 and Gram-positive bacterial proteases [115, 118, 122]. In fact, some pathogens secrete proteases allowing them to degrade adherent junctions and penetrate the physical barrier. Sensing microorganisms during the invasion process indirectly by their activity by Psh enables a rapid response against them [118]. Hence, Psh defines a parallel proteolytic cascade, independent of PRRs, which guards the *Drosophila* hemolymph against abnormal proteolytic activities.

Toll was first identified as an essential component in the dorsal-ventral embryonic development of the fruit-fly *D. melanogaster* [123]. Yet, in the context of early embryogenesis, a different serine protease cascade is involved including the CLIP proteases Gastrulation Defective and Snake and terminates with the protease Easter cleaving Spz instead of SPE [124-126]. Thus, SPE and Easter are terminal proteases of two different cascades involved in immunity and development, respectively, which commonly process Spz to activate the Toll pathway. SPE and Easter are activated differently, revealing that the Toll signaling pathway can be triggered in response to different cues and be used in different physiological processes.

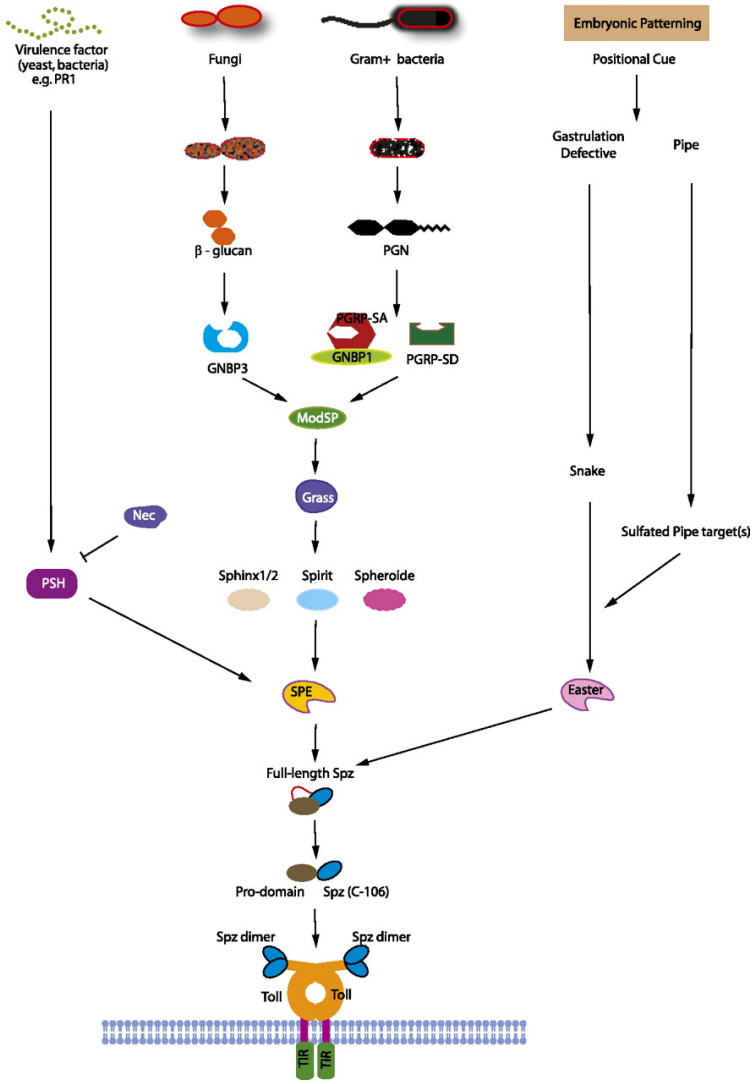

Figure 1. Toll pathway activation in *Drosophila*. Different serine protease cascades converge at Spz cleavage. The binding of the cleaved active Spz to Toll activates the receptor and initiates an intracellular signaling cascade [100].

c. Spätzle Structure, Secretion, and Activation

The activation of SPE by either of the two pathways (i.e. PRR-dependent or PRR- independent) leads to the cleavage of the *Drosophila* cytokine-like molecule Spz. Spz was originally identified in the *Drosophila* egg perivitelline compartment as an essential component for the establishment of embryonic dorsal-ventral polarity [127]. In

that context, Spz requires activation by a proteolytic processing reaction terminating by the protease Easter to bind to Toll and establish an intracellular signal transduction pathway involved in the dorsal-ventral patterning of the *Drosophila* embryo [125, 128]. Spz structure was shown to resemble mammalian growth factors and particularly the neurotrophin family such as the nerve growth factor [128].

Unlike in mammals where Toll-like receptors act as PRRs that directly recognize microbial components [129], *Drosophila* Toll needs to bind to the activated form of Spz to trigger a downstream signaling cascade. The *Drosophila* genome encodes nine Toll receptors [130] and six Spz genes [131], but only the canonical Toll-Spz or Toll1-Spz1 pathway in development and immunity have been well investigated. The interactions with one another and the associated functions of the remaining Toll and Spz are less well understood. The five other Spz genes appear to conserve the cystine-knot domain and retain a characteristic intron-exon structure found in the prototype Spz-1 gene, suggesting that these homologs may function as ligands for corresponding Toll receptors [131]. In one study, it was shown that in addition to Toll1, Toll5 was shown to activate the promoter of the antifungal peptide drosomycin, whereas no Toll other than Toll1 activated the expression of antibacterial peptides [130]. More recently, both Toll1 and Toll7 were shown to interact with Spz1, Spz2, and Spz5 leading to the activation of the *drosomycin* promoter [132, 133]. Spz4 was found to be strongly expressed in larvae and adult stages, similarly to Toll-5, suggesting that Spz4 may bind to Toll-5 in the context of fungal infections [131]. Other Toll/Spz interactions were shown to play roles unrelated to immunity such as Spz2 and Spz5 that interact with Toll-6 and Toll-7 promiscuously to regulate neurotrophism in *Drosophila* central nervous system [134].

The host protein Spz is initially synthesized as an inactive prepro-molecule that is processed internally at the endoplasmic reticulum by removing the N-terminal signal peptide before being secreted from the cells as a homodimeric disulfide-linked precursor molecule [124, 135]. The precursor is composed of two parts: a regulatory N-terminal prodomain (25 kDa) that can be of varying size depending on the splicing process [136] and a C-terminal domain (12 kDa) fragment of 106 amino acids (C-106) containing the signaling activity. Spz contains nine cysteine residues, seven of which are clustered in the active C-terminal domain, allowing the latter to adopt a dimeric cystine knot structure similar to the fold found in nerve growth factors [128, 137]. SPE cleaves Spz at amino acid number 106 from the C-terminal end, yielding the two fragments that remain non-covalently associated [128, 135]. Interestingly, even after cleavage, the two monomers remain associated through an interchain disulfide bond located in the C-terminal domain [135]. Only C-106 binds to Toll, whereas the prodomain seems to mask the receptor-binding site and thus prevent the interaction of Spz with Toll [135, 138]. Activation-induced proteolysis causes a conformational change that exposes determinants needed for binding to the Toll receptor [138]. The prodomain is then released from C-106 upon Toll binding [139].

The stoichiometry of binding of Spz to Toll has been controversial. One model implies that one Spz dimer binds to two Toll receptors (1:2). In that context, the binding of one C-106 dimer causes the cross-linking of two receptors and activates the Toll pathway [135], a mode of activation shared with other type I transmembrane receptors [140]. C-106 dimer binds to the N-terminal half of one Toll molecule resulting in a conformational change that causes the receptor to switch to an active conformation [141]. The second Toll molecule binds to C-106 with lower affinity, and this complex is

further stabilized by interactions between the C-terminal ectodomains of the receptors. This results in bringing the Toll intracellular domains into proximity, allowing them to transmit a signal. The complex is thus stabilized by both receptor-ligand and receptor-receptor interactions [141].

In a newer model, a stoichiometry of two Spz dimers-two Toll receptors (2:2) is reported. The binding of each of the two Spz dimers to the N-terminal end of one of the two Toll receptors triggers a conformational change in the receptors to activate downstream signaling. The binding of the mature Spz to Toll allows the interactions of two sites of the receptor ectodomains, one near the N-terminus and the other between the C-terminal juxtamembrane sequences, forming a heterotetrameric signaling complex [142].

d. Intracellular Signaling Cascade Activates the Transcription of Genes Encoding AMPs

Upon Spz recognition by Toll, a signaling complex is assembled at the cytoplasmic domain of Toll. Toll is an integral membrane protein with a large N-terminal extracellular domain characterized by leucine-rich repeats [123] flanked by cysteine-rich motifs [143]. The cytoplasmic intracellular domain shares significant similarities with the mammalian interleukin-1 receptor (IL-1-R), hence referred to as the Toll-IL-1-R (TIR) domain [144].

Through its TIR domain, Toll interacts with a heterotrimeric complex composed of MyD88, Tube, and Pelle [145]. MyD88 is an adaptor protein that associates both with Toll through its TIR domain and with Tube through its death domain [145-147]. Tube is a scaffolding protein that recruits Pelle to the complex by a distinct binding

surface on the Tube death domain, allowing a simultaneous association with MyD88 and Pelle [147-149]. Pelle, also having a death domain, is a serine-threonine kinase that is homologous to mammalian interleukin-1 receptor-associated kinases (IRAKs) [150]. Upon recruitment to the complex, Pelle undergoes autophosphorylation [151]. Then, although it has not been directly shown, Pelle seems to be the most probable kinase to phosphorylate Cactus, a *Drosophila* NFκB inhibitor, since it was found that its kinase activity is required for Cactus phosphorylation [152, 153]. In naive adult flies, Cactus is bound to the NF-κB transcription Dif, inhibiting its activity and limiting its nuclear translocation. Thus, Dif nuclear import requires Cactus degradation [154, 155], and this is achieved through its phosphorylation. Upon its phosphorylation at N-terminal serine residues, Cactus is ubiquitylated and degraded through the proteasome pathway [156, 157], freeing the transcription factor and allowing its translocation to the nucleus where it activates the transcription of hundreds of target genes including those encoding AMPs [100, 158].

Toll can activate two closely related NF-κB factors in a context-dependent manner. Dorsal was originally identified as an important morphogen in dorsal-ventral polarization in contrast to Dif (or Dorsal-related immunity factor), which was not found to participate in dorsal-ventral patterning. Dif, but not Dorsal, mediates Toll-dependent induction of immunity genes in *Drosophila* adults [159], whereas they exhibit overlapping immune functions in larvae [159, 160].

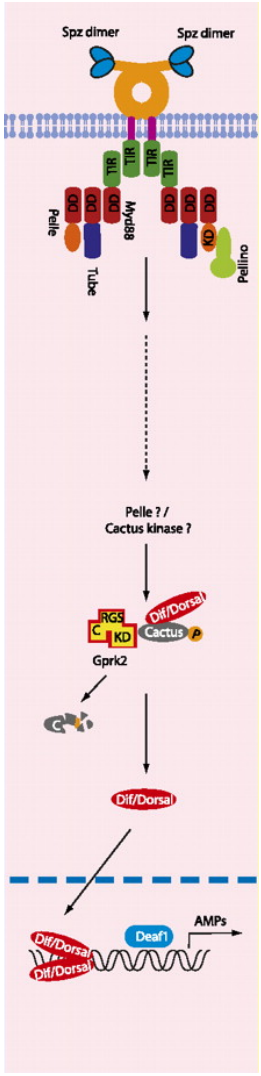

Figure 2. Toll intracellular signaling pathway in *Drosophila*. Toll activation leads to phosphorylation and degradation of Cactus, freeing the transcription factor Dif or Dorsal to translocate to the nucleus and activate the transcription of hundreds of genes [100].

2. *Characterization of Mosquito Toll Pathway*

The Toll immune signaling pathway is not yet fully characterized in *A. gambiae*. A comparison of *A. gambiae* and *Drosophila* genomes, specifically immunity gene contents, allowed the identification of mosquito homologs of the well-established Toll pathway of *Drosophila* [71, 82]. Immune-related gene families involved in pattern recognition, signal modulation, and effector systems were less conserved between the two organisms and showed species-specific expansions.

Seven distinct PGRPs have been identified in the *Anopheles* genome, with three of them belonging to the short subfamily (PGRP-S1, -S2, and -S3) and four belonging to the long subfamily (PGRP-LA, -LB, -LC, and -LD) [71]. Among these, PGRP-LC seems to play a central role in defense against systemic and oral bacterial infections [161]. PGRP-LC kd also increases mosquito susceptibility to *Plasmodium* parasites which could be an indirect effect of the changes in the size of the gut microbiota in this genotype. Three main isoforms of PGRP-LC gene have been detected with PGRP-LC2 being upregulated following immune and oxidative challenges (Gram-positive and Gram-negative bacteria, PGN and H_2O_2) in contrast to PGRP-LC1 which is induced by none, whereas PGRP-LC3 is upregulated only after challenge with Gram-positive and Gram-negative bacteria [71]. As for PGRP-LA isoforms, PGRP-LA1 responds to PGN and *E. coli*, whereas PGRP-LA2 only to *S. aur*. PGRP-LB is upregulated in adult mosquitoes following *Plasmodium* infection [162] and its expression remains elevated throughout the parasite's life cycle [71]. In addition, PGRP-S1 was identified as the only short PGRP to be induced by bacteria. Functional genetic studies in *Anopheles stephensi* revealed roles for PGRP-LD and PGRP-LA in regulating the homeostasis of the gut microbiota, whereby PGRP-LD but not PGRP-LA function was essential for promoting the structural integrity of the gut peritrophic matrix [163, 164].

In *Anopheles*, six GNBPs have been identified with four of them being mosquito-specific [71]. All of the members of the *A. gambiae GNBP* gene family were reported to be immune-responsive to varying pathogens, with both overlapping and unique functional attributes [165]. *GNBPA2, GNBPB1, GNBPB3,* and *GNBPB4* silencing differentially compromised mosquito resistance against bacteria, whereas *GNBPA2* and *GNBPB3* were also likely to play significant roles in the defense against

P. berghei and *P. falciparum* infections. Among all members, GNBPB4 displayed the most profound activity against bacteria and *Plasmodium* species, indicating that it is a major factor in the defense against a broad range of pathogens [165].

Our current understanding of the Toll pathway activation in *A. gambiae* remains largely unknown. None of the components involved in the protease cascade activating the Toll receptor in *Drosophila* seems to exhibit mosquito orthologs [82]. The *Drosophila* CLIP protease Persephone is also not conserved in the mosquito [71], suggesting that CLIPs have undergone significant diversification in insects. In addition, the CLIP protease responsible for Spz cleavage remains unidentified in the mosquito. The short and very specific SPE cleavage site recurs in the *A. gambiae* CLIPB5 [82], suggesting that it could be a possible candidate for Spz cleavage. Six Spz-like proteins have been identified in the *A. gambiae* genome, but their phylogenetic relationships with the respective homologs and their role in immunity remain unknown [71]. *A. gambiae* genome encodes 11 Toll genes [71], of which only four were shown to be orthologs of *Drosophila* counterparts [166]. Toll-11 is the only Toll receptor, so far, to be shown to be involved in immunity by providing partial resistance to *P. falciparum* [167]. In the dengue transmitting mosquito *Aedes aegypti*, the Spz1C-Toll5A axis seems to be analogous to the *Drosophila* Spz-Toll complex with respect to antifungal defense [168].

The Toll intracellular signaling pathway is conserved in *A. gambiae*. Mosquito orthologs of MyD88, Tube, Pelle, and Cactus have been identified [71]. One major difference in the intracellular components of the Toll pathway is the absence of an ortholog of Dif in *A. gambiae*. Thus, the mosquito genome encodes only two NF-kB transcription factors: Rel1 (previously known as Gambif-1 [169]) and Rel2, which are

orthologs of *Drosophila* Dorsal and Relish, respectively [71]. The infection-responsive activation of the Toll pathway leads to the nuclear translocation of Rel1, whereas the Imd pathway is controlled by Rel2. Both Rel1 and Rel2 play an important role in anti-*Plasmodium* defense. Depletion of Cactus, the negative regulator of Rel1, completely blocked *P. berghei* development [170], and reduced *P. falciparum* infection levels in *A. gambiae*, suggesting that Toll-Rel1 pathway may be more effective against *P. berghei* [171]. The depletion of Cactus also increased the basal expression of several anti-*Plasmodium* factors such as TEP1, LRIM1, and APL1C leading to complete prevention of *P. berghei* development in the mosquito [170, 171]. Both Rel1 and Rel2 control the expression of *A. gambiae* AMPs such as DEF1, CEC1, CEC3, and GAM1 [172].

D. Other Key Immune Signaling Pathways in Insects

In addition to the Toll signaling pathway, insects immune response is regulated by other evolutionary conserved signaling cascades such as the Imd and JAK/STAT pathways. These pathways are well characterized in *Drosophila* and were shown to confer resistance against an array of invading pathogens including bacteria, parasites, and viruses.

1. Imd pathway

The Imd pathway primarily governs defense reactions against Gram-negative bacteria. In *Drosophila*, the Imd pathway is triggered when the extracellular receptor PGRP-LC or the intracellular soluble receptor PGRP-LE bind meso-diaminopimelic acid (DAP)-type PGN [173], which comprises the cell wall of Gram-negative bacteria

and certain Gram-positive strains. A more recent study revealed that PGRP-SD acts upstream of PGRP-LC to activate the Imd pathway by enhancing the localization of PGN to the cell surface [174]. Once bound to PGN, the receptors recruit a signaling complex consisting of a death domain protein Imd, the adaptor protein FADD, and the caspase-8 homolog DREDD. DREDD cleaves Imd, creating a novel binding site for the E3-ligase inhibitor of apoptosis 2, Iap2, which K63-ubiquitinate Imd [175]. The K63-polyubiquitin chains are thought to recruit and activate a complex composed of the transforming growth factor β-activated kinase 1, TAK1, and TAK1-associated binding protein 2, TAB2. The TAK1/TAB2 complex is responsible for the activation of the *Drosophila* IKK complex, which phosphorylates the transcription factor Relish, allowing its translocation into the nucleus where it activates the transcription of genes coding for anti-Gram-negative peptides such as drosocins and diptericins [176-179]. DREDD is also required for mediating the cleavage of Relish [180]. Caspar acts as a negative regulator of the Imd pathway by inhibiting DREDD-mediated cleavage of Relish [181]. Relish consists of Rel homology domain (RHD) and inhibitory ankyrin repeat domains (ARD) [182]. Activation of Relish by phosphorylation and signal-induced endoproteolytic cleavage frees RHD from ARD, followed by its translocation into the nucleus, interaction with DNA, and initiation of the transcription of target genes (reviewed in [101]).

Most of the components of the Imd pathway are conserved in *Anopheles* [71], however, the pathway in mosquitoes remains poorly characterized and its involvement in immunity has been mainly concluded from functional genetic studies of its key transcription factor Rel2. The *Anopheles* ortholog of Relish, Rel2, is involved in anti-bacterial and anti-*Plasmodium* defense. Rel2 produces two isoforms through alternative

splicing: Rel2-F, that encodes a full-length protein containing both RHD and ARD domains, and Rel2-S, that encodes only RHD and is thus constitutively active to confer basal immune functions. Rel2-F and Rel2-S isoforms were shown to be involved in defense against *S. aureus* and *E. coli*, respectively [172]. In addition, silencing of Rel2 increased the numbers of *P. berghei* oocysts [172], while depletion of Caspar completely blocked *P. falciparum* ookinetes development in three major *Anopheles* malaria vector species and reduced the numbers of *P. berghei* oocysts [171], indicating the critical role of Imd-Rel2 pathway against the human *Plasmodium* parasite, whereas Toll-Rel1 pathway was shown to be more effective against the rodent parasite. Indeed, Rel2 overexpressing transgenic *A. stephensi* mosquitoes were more resistant to *P. falciparum* than *P. berghei* [183].

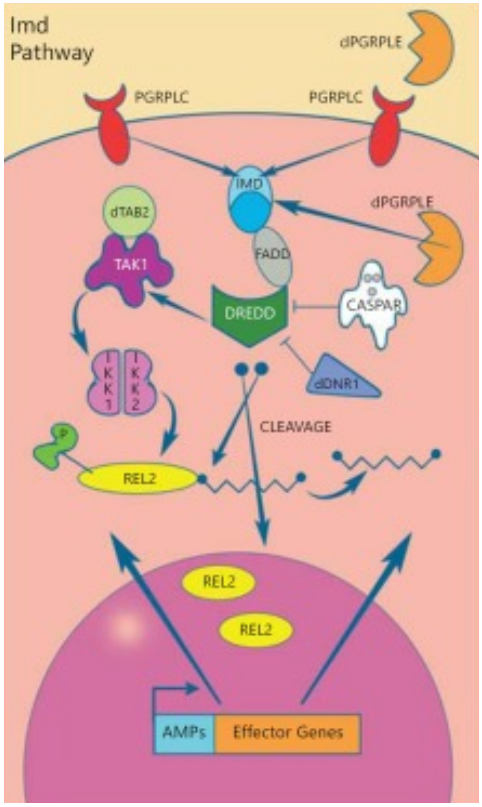

Figure 3. Imd pathway in *Anopheles* mosquito. Upon recognition of pathogens by PGRP-LC or PGRP-LE receptors, a signaling cascade is initiated, resulting in the cleavage and translocation of active Rel2 into the nucleus to regulate the transcription of target immune genes [218].

2. JAK/STAT Pathway

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway was first recognized for its role in a variety of developmental processes in *Drosophila*, and later for its implication in antibacterial and antiviral defense [102, 184]. In *Drosophila*, this signaling pathway is triggered by the binding of the cytokine ligand Unpaired (Upd) to the transmembrane receptor Domeless (Dome), causing the receptor-associated JAK Hopscotch to phosphorylate both itself and Dome to create docking sites for STAT92E. STAT92E is then phosphorylated, dimerized, and translocated to the nucleus, where it induces target gene expression (reviewed in [185]).

There are two STAT genes in *A. gambiae* (*Ag*STAT-A and *Ag*STAT-B) which appear to have originated by gene duplication [71] and a one-to-one orthology relationship exists for JAK and Dome in the two species [186]. Recent studies have linked this pathway to anti-*Plasmodium* defense. *Ag*STAT-B was previously reported to translocate to the nucleus of fat body cells in response to bacterial infection [187] and was later shown to regulate the expression of *Ag*STAT-A mRNA [188]. *Ag*STAT-A induces the expression of nitric oxide synthase (NOS) following bacterial and *Plasmodium* infection. While this pathway is not essential for mosquitoes to survive a bacterial challenge, it markedly reduces the number of *Plasmodium* oocysts in the midgut. Silencing of *Ag*-STAT-A significantly enhanced *P. berghei and P. falciparum* oocyst development. Depletion of SOCS, a negative regulator that prevents STAT phosphorylation, increased NOS levels decreasing the number of early oocysts. In addition, chemical inhibition of NOS activity after oocyst formation promoted oocyst survival in infected mosquitoes. Altogether, these results suggest that the JAK/STAT

pathway induces a late-phase anti-*Plasmodium* response to the developing oocysts [188].

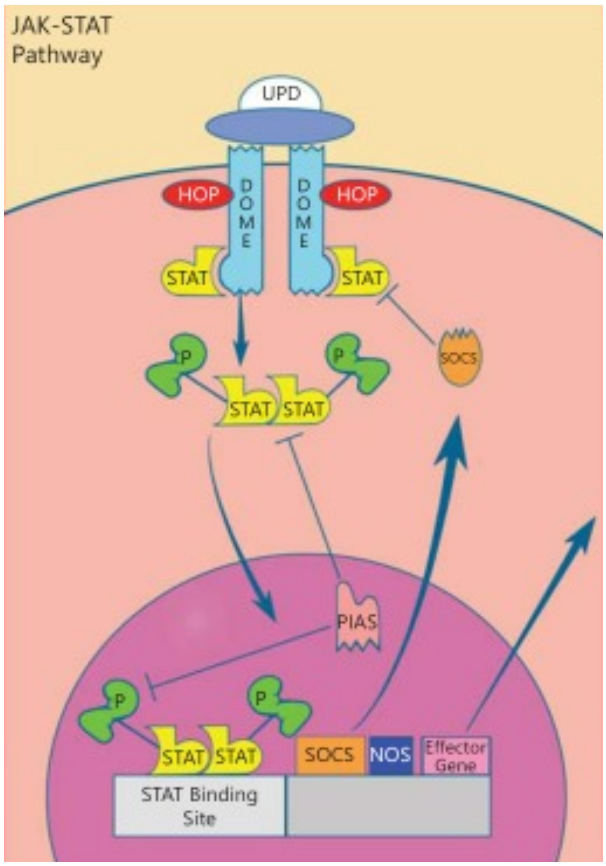

Figure 4. JAK/STAT pathway in *Anopheles* mosquito. The binding of the ligand Upd to Dome receptors leads to the eventual nuclear translocation of STAT and transcriptional activation of immune effector genes [218].

E. Specific Aims

Unlike *Drosophila*, our knowledge of the Toll pathway activation in *A. gambiae* remains largely fragmented, especially that there is no pathway-specific readout available. A better understanding of the activation of the mosquito Toll pathway and its contribution to mosquito immunity awaits rigorous functional genetic analysis of its upstream components specifically CLIPs, Toll, and Spätzle genes, and the identification of a pathway-specific readout. Such studies will also help identify whether functional

interactions exist between Toll and the complement-like pathway triggered by TEP1 which constitutes so far the hallmark of mosquito humoral responses to a broad range of microbes including malaria parasites, Gram-negative bacteria, and fungi. Furthermore, fungi are being considered promising biopesticides for the control of mosquito vectors of diseases [189-191], thus a better characterization of the Toll pathway is needed to better understand the potential mechanisms that may be used by fungi to evade mosquito immune responses.

In this project, we aim to characterize at the functional level the Spz gene family in *A. gambiae*, the major African vector of malaria. In this context, we will investigate the role of Spz genes in mosquito resistance and tolerance to bacterial (both Gram-positive and Gram-negative) and fungal infections using RNAi-based functional genetic screen. We will also study the expression of Spz genes in response to septic infections by bacteria and fungi to identify infection-responsive Spz genes.

CHAPTER II

MATERIALS AND METHODS

A. *Anopheles gambiae* rearing

All experiments were performed with 1-3 day old adult female *Anopheles gambiae* G3 strain mosquitoes, reared in a dedicated insectary in the Department of Biology at the American University of Beirut. Mosquitoes were maintained at 27 (±1) °C and 75 (±5) % humidity with a 12-hour day-night cycle. Larvae were reared in 752 cm^2 plastic pans at a density of approximately 150 larvae per pan and given Tetra® tropical fish food. Freshly emerged adult mosquitoes were collected from larval pans using a vacuum collector and fed on sugar pads containing 10% sucrose. To maintain the cycle, adult mosquitoes were given a mouse blood meal once per week to lay eggs; BALB/c mice were anesthetized with a solution of ketamine and xylazine, then placed on top of mosquito cages allowing the starved mosquitoes to feed on mice blood for approximately 15 minutes in total darkness.

B. Double-stranded RNA Synthesis and Gene Silencing by RNA Interference

Double-stranded RNA (dsRNA) synthesis was performed using the T7 RiboMax Express Large Scale RNA Production System (Promega) according to the manufacturer's instructions. Primers used for dsRNA productions are listed in Table 1 below. Transcription reactions were performed using 1 µg of linear DNA template and samples were incubated at 37°C overnight. RNase-free DNase was used to a concentration of 1 unit/µg of template DNA to degrade any DNA traces. All dsRNAs were purified with one volume of phenol:chloroform:isoamyl alcohol. Samples were

centrifuged for 2 minutes at 14000 rpm at room temperature, and the upper aqueous layer was then purified with one volume of chloroform:isoamyl alcohol. The upper aqueous layer was transferred to a new Eppendorf tube to which isopropanol was added (70% of total volume). Samples were left at -20^{o}C for 2 hours to allow RNA precipitation. Samples were then centrifuged for 30 minutes at 17000 g at 4^{o}C, and pellets were washed with 70% ethanol, air-dried, and resuspended in nuclease-free water to reach a final dsRNA concentration of 3.5 µg/µl.

In vivo gene silencing was performed as previously described [73]. A microinjector (Nanoject II, Drummond) was used to introduce 69 nl of a 3.5 µg/µl solution of gene-specific dsRNA in the thorax of female adult mosquitoes. Mosquitoes were anesthetized using CO_2 during injections and allowed to recover for 3-4 days before subjecting them to microbial challenges.

Table 1. Sequence of T7 primers used for dsRNA production

T7-LacZ-F	5'-TAATACGACTCACTATAGGGAGAATCCGACGGGTTGTTACT-3'
T7-LacZ-R	5'-TAATACGACTCACTATAGGGCACCACGCTCATCGATAATTT-3'
T7-Rel1-F	5'- TAATACGACTCACTATAGATCAACAGCACGACGATGAG-3’
T7-Rel1-R	5'- TAATACGACTCACTATAGTCGAAAAAGCGCACCTTAAT-3′
T7-TEP1-F	5'-TAATACGACTCACTATAGGGTTTGTGGGCCTTAAAGCGCTG-3'
T7-TEP1-R	5'- TAATACGACTCACTATAGGGACCACGTAACCGCTCGGTAAG-3'
T7-Spz1-F	5’-TAATACGACTCACTATAGGGCTTCCGAAAGGACTTTGGCA-3’
T7-Spz1-R	5’-TAATACGACTCACTATAGGGCGGTGGACTGCTGCTCCTGT-3’
T7-Spz2-F	5’-TAATACGACTCACTATAGGGGTGCAGATCTACAACTACCA-3’
T7-Spz2-R	5’-TAATACGACTCACTATAGGGCTCGTCGACCTGATCGAACT-3’
T7-Spz3-F	5’-TAATACGACTCACTATAGGGGGTGACTCCGATTGTTGGGA-3’
T7-Spz3-R	5’-TAATACGACTCACTATAGGGGCAGAACAGTTGATGGTACT-3’
T7-Spz4-F	5’-TAATACGACTCACTATAGGGGATATCGATCGTGCGACGGA-3’
T7-Spz4-R	5’-TAATACGACTCACTATAGGGGTGGTAGCGCTGGTTGTGGT-3’
T7-Spz5-F	5’-TAATACGACTCACTATAGGGCCGATTATCCTGCCTACCTT-3’
T7-Spz5-R	5’-TAATACGACTCACTATAGGGTACCGTGCCGAGTGATTGTT-3’
T7-Spz6-F	5’-TAATACGACTCACTATAGGGCGATGAAGCAGAACGTACTA-3’
T7-Spz6-R	5’-TAATACGACTCACTATAGGGCCGGTGCCTCTTCGCGTCTT-3’

The efficiency of gene silencing by RNAi was measured by quantitative Real-time PCR (qRT-PCR). Total nucleic acid was isolated using TRIzol reagent from 15 mosquitoes per Spz gene knockdown as follows. Each batch of 15 mosquitoes was ground in a 1.5 ml Eppendorf tube containing 500 µl TRIzol using a pellet pestle motor. A volume of 100 µl chloroform was added to each tube and vortexed to mix well then centrifuged for 15 minutes at 12000 rpm at 4°C. The top aqueous layer was transferred to a new tube, and isopropanol (70% final concentration) was added to precipitate the total nucleic acid, and tubes were centrifuged for 30 minutes at 12000 rpm at 4°C. Pellets were washed once with 70% ethanol, air-dried, and resuspended in nuclease-free water. DNA removal from RNA samples was performed by incubation for 30 minutes at 37°C with RNAse-free DNAseI (Thermo Scientific) according to the manufacturer's instructions. RNAs were extracted with 1:1 ratio phenol:chloroform:isoamyl alcohol, precipitated using isopropanol, washed with 70% ethanol, and resuspended in nuclease-free water. cDNA synthesis was performed with 1 µg of DNAseI-treated RNA as a template using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. The synthesized cDNAs were used as templates for qRT-PCR at a working dilution of 1/5 in nuclease-free water. qRT-PCR was performed in a CFX96 Real-Time Detection System (Bio-Rad) using QuantiNova SYBR Green PCR (Qiagen) according to the manufacturer's instructions. The reaction starts at 95°C for 10 seconds to denature dsDNA, then the temperature is lowered to 60°C for 30 seconds to allow primers annealing and template extension, and the cycle is repeated 39 more times. Primers used to score the efficiency of gene silencing are listed in Table 2. The *A. gambiae* ribosomal S7 gene was used as an internal control for normalization using the primers, AgS7-F: 5'-AGAACCAGCAGACCACCATC-3'and AgS7-R: 5'-

GCTGCAAACTTCGGCTATTC-3'. Relative gene expression values were calculated using the comparative C_T method after checking for the efficiency of target amplification. Three independent experiments were performed for each gene knockdown and statistical analysis was performed using the paired student *t*-test.

Table 2. Sequence of Spz primers used in qRT-PCR

Spz1-rt-F	5'-TACGAACGAGCCGGGAAAGA-3'
Spz1-rt-R	5'-AGGTCCGCGTACTTGTCCATA-3'
Spz2-rt-F	5'-GACCAGAGCATTGCCGAGAC-3'
Spz2-rt-R	5'-TCGACGGCGATGTTTTTCGAT-3'
Spz3-rt-F	5'-TGCGTGCGAATCGAAGATCG-3'
Spz3-rt-R	5'-CACACTTCCTGGTGGATCGC-3'
Spz4-rt-F	5'-TTCCGACTGGTTCCGGTTTC-3'
Spz4-rt-R	5'-AGCTCGATCGACTCCTTGGT-3'
Spz5-rt-F	5'-ACATTACACCGCAGACGGCA-3'
Spz5-rt-R	5'-GCACTCCGTTGACGCACAAA-3'
Spz6-rt-F	5'-CTCCCGACCGACAACGTGTA-3'
Spz6-rt-R	5'-GCTGATTAGCCTTGGCACTGG-3'

C. Microbial Survival Assays

Fifty to sixty freshly emerged adult female mosquitoes were injected with dsRNAs complementary to each of the six Spätzle genes (Spz1-6) to trigger silencing by RNAi. Mosquitoes injected with *dsLacZ* (complementary to the β-galactosidase gene) served as controls. The survival of dsRNA-treated mosquitoes was scored over a period of two weeks after an intrathoracic injection of a suspension of GFP-expressing *Serratia marcescens* DB11 strain (OD_{600}=0.0005) [192] or chloramphenicol-resistant GFP expressing *Staphylococcus aureus* strain RN4220 (OD_{600}=0.8) [193] in PBS, or after spraying with a suspension of *Beauveria bassiana* (strain 80.2) containing $1x10^8$ conidia/ml in 0.05% Tween-80, prepared as previously described [40, 194]. Briefly, a 3-4 week-old lawn of *B. bassiana* growing on potato dextrose agar was scraped and

filtered using glass wool to harvest the spores and separate them from other mycelial structures. The solution was then centrifuged for 10 minutes at 4000 g at 4ºC, and the pellet was washed with deionized water and suspended in 500 µL of 0.05% Tween-80 solution. A hematocytometer was used to estimate spore counts. At least 4 biological experiments were performed for each *Spz* gene knockdown using different batches of mosquitoes and spores. The survival rates and statistical significance in the different genotypes were calculated using the Kaplan Meier survival analysis and the log-rank test in GraphPad Prism, version 6.

D. Microbial Proliferation Assays

The role of Spz genes in resistance to septic infections was determined by scoring the Colony Forming Units (CFUs) in whole mosquitoes. *DsSpz1-6, dsLacZ* (negative control), and *dsRel1* (positive control) mosquitoes were injected intrathoracically with 69 nl of *S. aureus* strain RN4220 (OD_{600}=0.8) and CFUs were scored in whole mosquito lysates at 24 hours post-infection, using the spread plate method, as previously described [88, 89]. Briefly, for each genotype, at least 5 batches of eight mosquitoes each were ground in 500 µl Luria Bertani (LB) broth. Then, serial dilutions of mosquito homogenates were plated onto LB agar plates supplemented with chloramphenicol, and the CFUs were scored after 24 hours. Since the *S. aureus* strain RN4220 expresses GFP, colonies were counted under a fluorescent microscope to avoid the accidental counting of potential chloramphenicol resistant contaminating bacteria. At least 4 biological experiments were performed for each *Spz* gene knockdown. Statistical significance was calculated using the Mann-Whitney test. Medians were considered significantly different if $P<0.05$.

The proliferation of *B. bassiana* in spore-infected mosquitoes was scored by qRT-PCR as follows. Briefly, *dsLacZ*, *dsTEP1,* and *dsSpz1-6*-treated mosquitoes were injected each with approximately 30 *B. bassiana* spores in nanopure water. Four days later, 15-20 whole mosquitoes were ground in liquid nitrogen with a mortar and pestle to create a fine powder. The powder was collected into Eppendorf tubes and genomic DNA was extracted using 200 µl of CTAB buffer (0.1M Tris-HCl, pH 8.0, 0.01M EDTA, 1.4M NaCl, 2% cetyltrimethyl ammonium bromide). Samples were heated for 5 minutes at 65ºC and DNA was then purified with a 1:1 volume of chloroform. Samples were centrifuged for 5 minutes at 12000 rpm at room temperature, and the upper aqueous layer was transferred to a new Eppendorf tube where DNA was precipitated at -20ºC using isopropanol (70% of total volume). After centrifugation for 15 minutes at 12000 rpm at room temperature, pellets were washed with 70% ethanol, air-dried, and resuspended in nuclease-free water. QRT-PCR was performed in a CFX96 Real-Time Detection System (Bio-Rad) using the SYBR Green JumpStart^{TM} Taq ReadyMix (Sigma-Aldrich) according to the manufacturer's instructions. *B. bassiana* primers used in qRT-PCR are the following: Bb_ITSII_F: 5'-GCC GGC CCT GAA ATG G-3'and Bb_ITSII_R: 5'- GAT TCG AGG TCA ACG TTC AGA AG-3 [195]. The *A. gambiae* ribosomal S7 gene was used as an internal control for normalization using the primers: AgS7-F: 5'-AGAACCAGCAGACCACCATC-3'and AgS7-R: 5'GCTGCAAACTTCGGCTATTC-3'[41]. Relative gene expression values were calculated using the comparative C_T method after checking for the efficiency of target amplification.

E. RNA Extraction, Reverse Transcription, and Real-Time PCR

To determine the expression profiles of Spz genes in response to septic infections, total RNA was extracted from whole mosquitoes using the NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's instructions. RNA was extracted from whole mosquitoes at 6, 12, and 24 hours post *S. aureus* bacterial infection and at 24, 48, and 72 hours post spraying with *B. bassiana*. PBS-injected mosquitoes at the indicated bacterial time points and naïve mosquitoes served as controls.

For cDNA synthesis, 1 μg of extracted RNA was reverse transcribed using the iScript cDNA synthesis (Biorad) according to the manufacturer's protocol. A 1/5 dilution of synthesized cDNAs in nuclease-free water was prepared and 1 μl of the diluted samples was added to the qRT-PCR reaction. qRT-PCR was performed in a CFX96 Real-Time Detection System (Bio-Rad) using QuantiNova SYBR Green PCR (Qiagen) according to the manufacturer's instructions. Primers used are listed in Table 2. The *A. gambiae* ribosomal S7 gene was used as an internal control for normalization using the primers, AgS7-F: 5'-AGAACCAGCAGACCACCATC-3'and AgS7-R: 5'-GCTGCAAACTTCGGCTATTC-3'. Two independent experiments were performed. Relative gene expression values were calculated using the comparative C_T method. Statistical analysis was performed using the student's t-test.

CHAPTER III

RESULTS

A. Silencing of Spz genes by RNA interference

The discovery of RNAi in the mid '90s and its effective application in studying gene function have revolutionized functional genetic studies in several organisms [196-198]. In this project, we utilized RNAi to study the function of Spz1-6 genes in mosquito defense against microbial infections. Silencing was achieved by the injection of Spz gene-specific dsRNA into the thorax of adult female mosquitoes. Gene silencing efficiency was scored at three days post dsRNA injections by qRT-PCR. The efficiency of Spz1-6 silencing in our hands was 67, 37, 57, 41, 51, and 61%, respectively (Fig. 5).

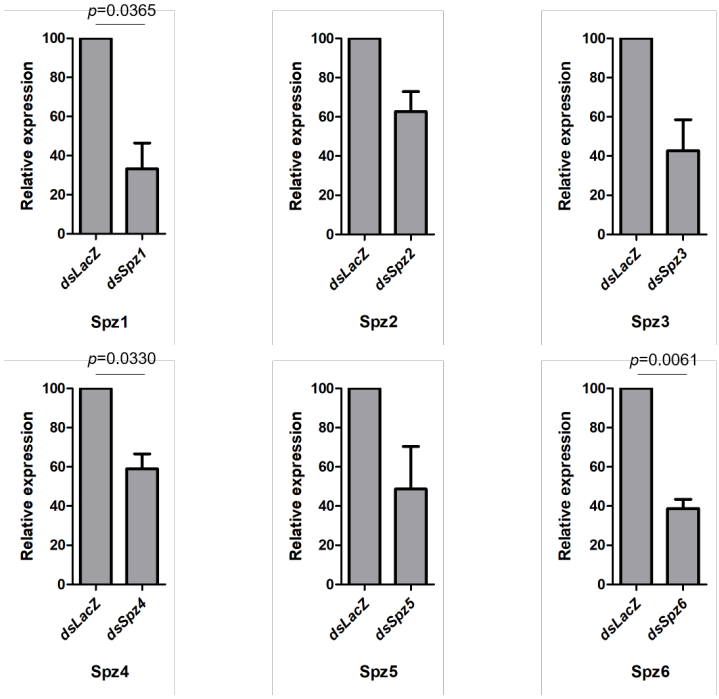

Figure 5. *Spz1-6* knockdown efficiency after silencing by RNAi. Transcript levels of Spz1-6 were measured by qRT-PCR in whole female mosquitoes at 3 days following injection of their respective dsRNA. Error bars represent standard error of the mean of three biological repeats. Statistical analysis was done using the Student's *t*-test.

B. Spz gene silencing shows complex phenotypes with respect to mosquito tolerance to bacterial and fungal infections

Recent studies suggest that the ability of an organism to survive an infection is determined by more than just the capacity to demonstrate physiological resistance by eliminating the invading microorganism [199]. Rather, tolerance, defined as the ability to limit the health impact and fitness effects caused by an infection, may be used in conjunction with resistance to promote host survival to a given infection. The survival rate of an organism following an infection is considered one of the key indicators of tolerance. To identify the role of Spz genes on mosquito tolerance, adult female *A. gambiae* mosquitoes were first injected with gene-specific dsRNAs for Spz1-6 to trigger silencing by RNAi, then their survival rates were scored over a period of two weeks after injection of the Gram-positive bacterium *S. aureus* (OD_{600}=0.8) or the Gram-negative bacterium *S. marcescens* (OD_{600}=0.0005) or after spraying with a suspension of the entomopathogenic fungus *B. bassiana* ($1x10^8$ conidia/ml). Mosquitoes treated with ds*LacZ* (dsRNA specific to the β-galactosidase gene) were used as control. *Spz1-6* kds revealed mixed survival phenotypes after infections with *S. aureus* (Fig. 6, Table 3) and *B. bassiana* (Fig. 7, Table 4), which do not permit to draw clear conclusions on the role of Spz genes in mosquito tolerance to infections with Gram-positive bacteria and fungi. For instance, in the context of *S. aureus* infections, *Spz2* and *Spz4* kds gave significant phenotypes in 4 out of 8 different trials, whereas the other Spz genes showed significant phenotypes in fewer trials (Table 3). Similarly, in the context of *B. bassiana* infections, *Spz2* kd showed significant survival phenotypes in 6 out of 8 trials whereas the rest of the Spz genes showed significant RNAi phenotypes in only few trials (Table 4), which suggests that Spz2 may be a key player in the activation of the Toll pathway in response to fungal and Gram-positive bacterial

infections. However, in the context of *S. marcescens* infections, none of the Spz gene kds revealed a significant survival phenotype (Fig. 8).

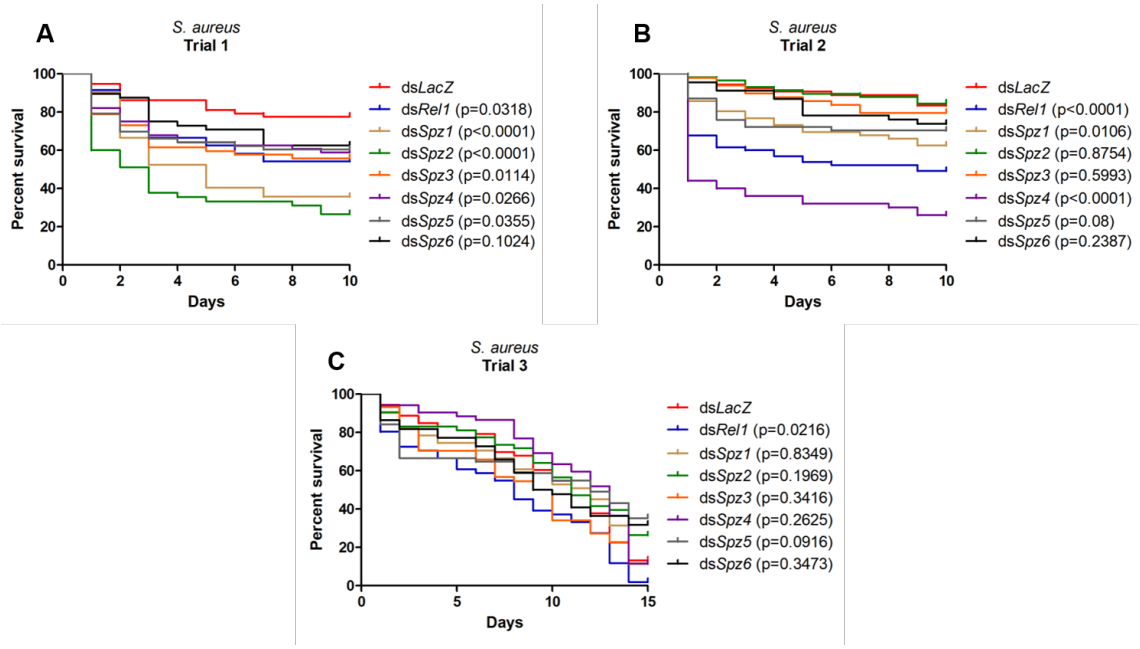

Figure 6. *Spz1-6* knockdowns show mixed survival phenotypes after infection with Gram-positive bacteria. (A-C) Survival assays of the indicated mosquito genotypes following injection with *S. aureus* (OD600= 0.8). Three independent biological experiments are shown. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test.

Table 3. Summary of Spz RNAi survival phenotypes after *S. aureus* infections. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test.

	ds*Rel1*	ds*Spz1*	ds*Spz2*	ds*Spz3*	ds*Spz4*	ds*Spz5*	ds*Spz6*
Trial 1	+	+++	+++	+	+	+	ns
Trial 2	+++	+	ns	ns	+++	ns	ns
Trial 3	+	ns	ns	ns	ns	ns	ns
Trial 4	ns	ns	ns	ns	ns	ns	ns
Trial 5	ns	++	++	++	+	+++	+++
Trial 6	ns	ns	+	ns	++	ns	++
Trial 7	ns	ns	++	ns	ns	ns	++
Trial 8	ns	ns	ns	ns	ns	ns	ns

ns, non-significant ($p > 0.05$); +, $p \leq 0.05$; ++, $p \leq 0.01$; +++, $p \leq 0.001$.

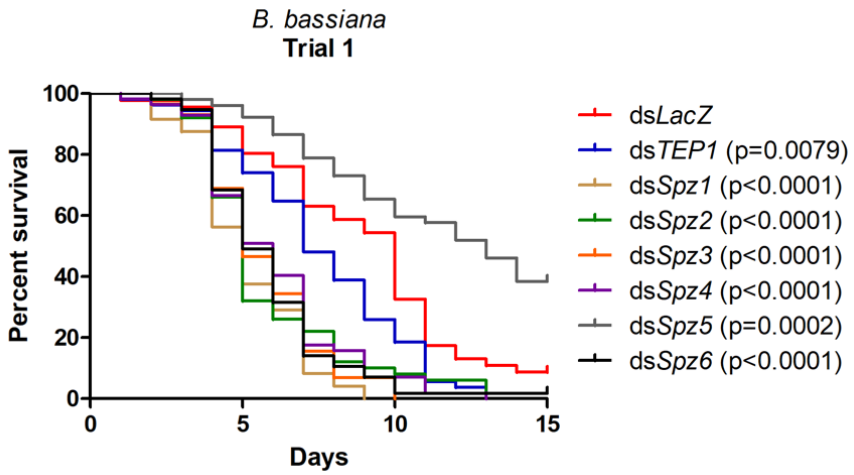

Figure 7. *Spz1-6* gene silencing reveals mixed phenotypes with respect to mosquito tolerance to fungal infections. Survival assay of the indicated mosquito genotypes after spraying with a *B. bassiana* suspension of 1x108 spores/ml. One biological experiment is is shown. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test.

Table 4. Summary of Spz RNAi survival phenotypes after *B. bassiana* infections. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test.

	ds*TEP1*	ds*Spz1*	ds*Spz2*	ds*Spz3*	ds*Spz4*	ds*Spz5*	ds*Spz6*
Trial 1	++	+++	+++	+++	+++	+++	+++
Trial 2	ns	ns	++	ns	+++	+	+++
Trial 3	ns	ns	++	ns	ns	ns	ns
Trial 4	ns	+	++	ns	+++	+++	+++
Trial 5	ns	ns	+	+	ns	ns	ns
Trial 6	ns	ns	++	++	ns	+	+
Trial 7	ns	ns	ns	ns	ns	ns	ns
Trial 8	ns	ns	ns	ns	ns	ns	ns

ns, non-significant ($p > 0.05$); +, $p \leq 0.05$; ++, $p \leq 0.01$; +++, $p \leq 0.001$.

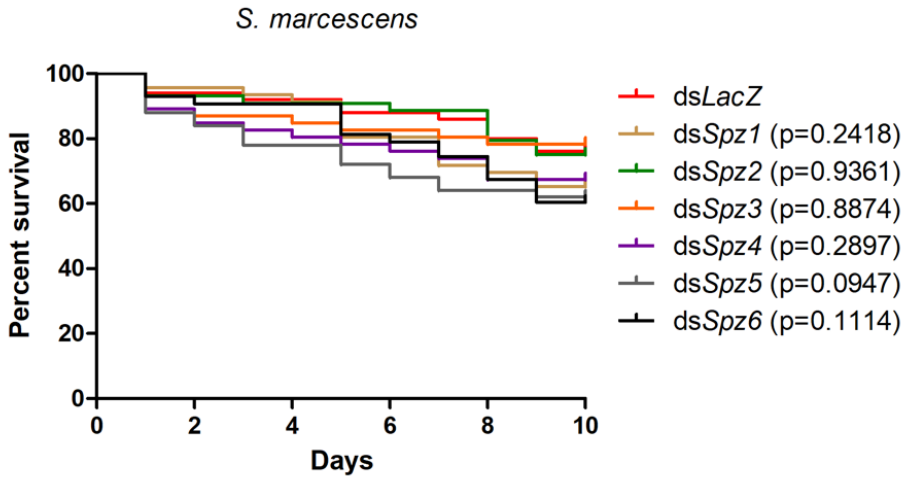

Figure 8. *Spz1-6* knockdown mosquitoes are not susceptible to Gram-negative bacterial infections. Survival assay of the indicated mosquito genotypes following injection with *S. marcescens* (OD600=0.0005). One representative experiment is shown from three independent biological experiments. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test.

C. Spz gene silencing has no significant effect on mosquito resistance to bacterial and fungal infections

Resistance is considered a direct measure of immune competence and is defined as the ability of an organism to clear microbes from the tissues. To address whether the Spz genes contribute to mosquito resistance to bacterial infection, ds*Spz1-6* and ds*LacZ* mosquitoes were injected with *S. aureus* (OD_{600}=0.8) and colony forming units (CFUs) were scored in whole mosquito lysates at 24 hours post-infection. Silencing *Spz1-6* did not alter host resistance to *S. aureus* infections as no significant difference in the CFU counts was observed between *Spz1-6* kd and control mosquitoes (Fig. 9). In addition, preliminary data (not shown) of the relative quantification of *B. bassiana* genomic DNA by qRT-PCR showed that fungal proliferation was not significantly enhanced in *Spz* kd mosquitoes in comparison to control mosquitoes, suggesting that *Spz* silencing may not have a significant effect on mosquito resistance to fungal infections.

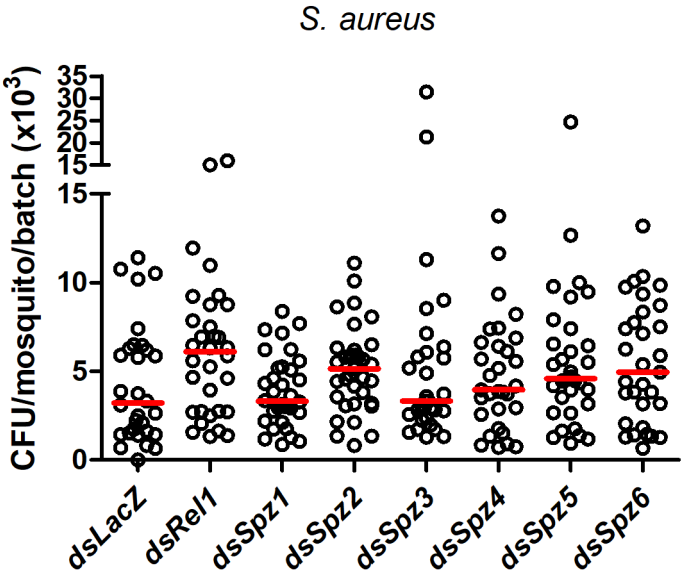

Figure 9. *Spz1-6* knockdown does not alter mosquito tolerance to systemic bacterial infections. Bacterial proliferation assays conducted on mosquitoes injected with *S. aureus* (OD_{600nm} = 0.8). Batches of 8 whole mosquitoes were ground in LB medium 24 hours after infection, and colony forming units (CFUs) were scored on LB plates supplemented with chloramphenicol. Each point on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using Kruskal Wallis test. Medians (red lines) were considered significant if $p < 0.05$. Data shown are from six independent biological experiments.

D. Spz expression is induced upon fungal, but not bacterial infections

The genes of the Spz-Toll-Cactus cassette are expressed in *Drosophila* adults, and their expression is upregulated in response to bacterial challenge [200]. Similarly, *Spz* expression is induced after immune challenge in the mealworm *Tenebrio molitor* [201], brine shrimp *Artemia sinica* [202], white-leg shrimp *Litopenaeus vannamei* [203], and the red palm weevil *Rhynchophorus ferrugineus* [204]. To address whether *Spz1-6* genes are under infection-induced transcriptional control in *A. gambiae*, their expression was quantified by qRT-PCR at 6, 12, and 24 hours after challenging mosquitoes with *S. aureus* (OD_{600}=0.8), or at 24, 48, and 72 hours after spraying with a suspension of *B. bassiana* ($1x10^8$ conidia/ml). Mosquitoes injected with sterile PBS and

sacrificed at the same time points as challenged mosquitoes, as well as naïve (non-infected) mosquitoes served as controls. All *Spz* transcript levels increased, to varying degrees, after challenge with *B. bassiana* in comparison to those in naïve mosquitoes, however only two genes, Spz1 and Spz5, showed a statistically significant upregulation at the 48-hour time point with a 2- and 2.65-folds increase, respectively (Fig. 10). Surprisingly, there was no significant effect of *S. aureus* infection on Spz expression. There was no significant difference in the relative expression of all *Spz* genes between infected and sterile PBS injected samples at the different time points in comparison to that in naïve mosquitoes (Fig. 11). In fact, almost all *Spz* expression values fell below the baseline.

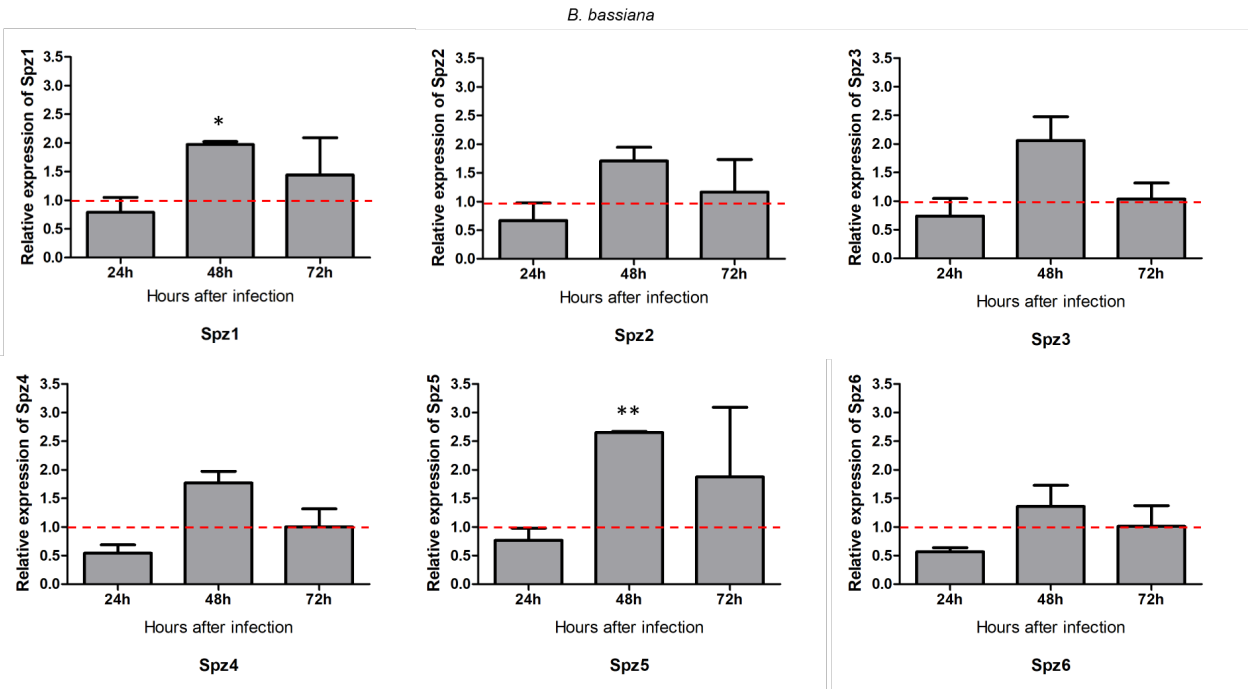

Figure 10. *Spz1-6* expression is upregulated after fungal infections. Expression profile of *Spz1-6* in whole mosquitoes 24, 48, and 72 hours after natural infection with *B. bassiana* ($1x10^8$ spores/ml). The results are based on two independent experiments and shown as mean values ± standard error of the mean (SEM). The naïve (non-infected) mosquitoes served as controls and their mean gene expression was adjusted to 1 as represented by the red dashed line. Statistical significance was calculated using the Student's *t*-test (*indicates $p < 0.05$, **indicates $p < 0.01$).

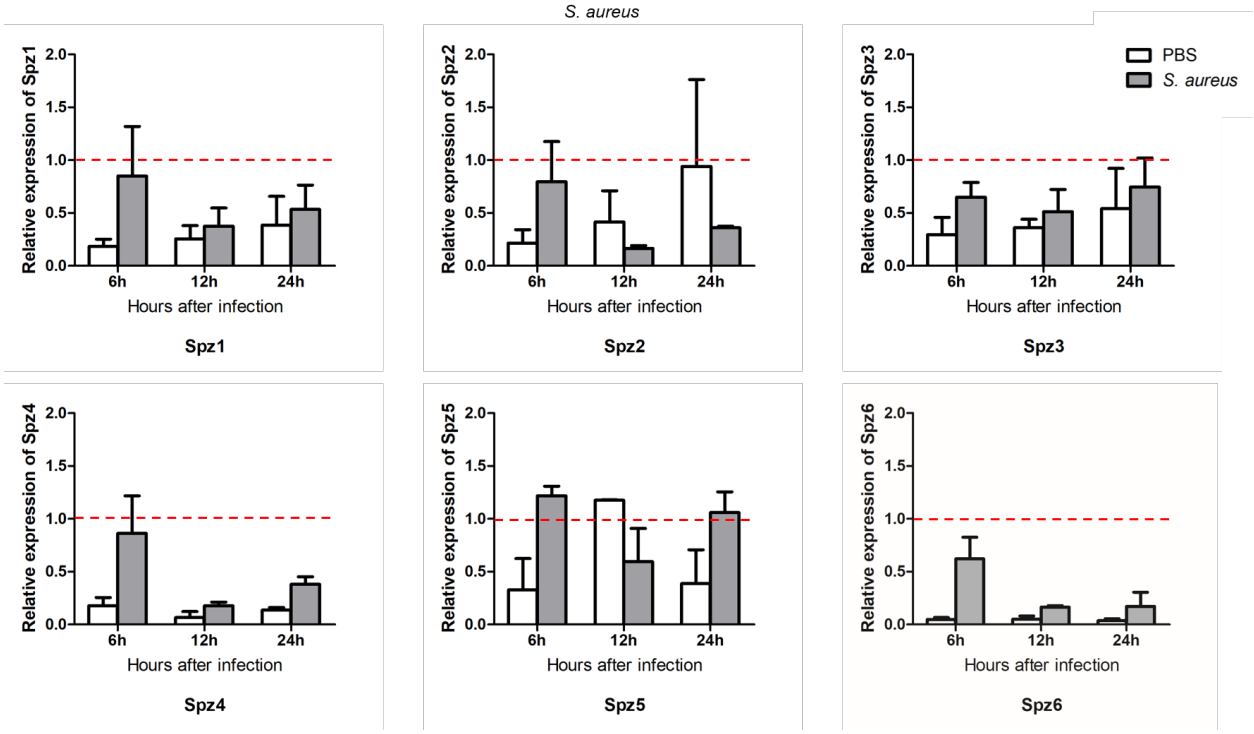

Figure 11. *Spz1-6* expression is not induced after Gram-positive bacterial challenge. Expression profile of *Spz1-6* in whole mosquitoes 6, 12, and 24 hours after infection with *S. aureus* (OD_{600}=0.8). The results are based on two independent experiments and shown as mean values ± standard error of the mean (SEM). The naïve (non-infected) mosquitoes served as controls and their mean gene expression was adjusted to 1 as represented by the red dashed line. Statistical significance was calculated using the Student's *t*-test and means were considered significantly different if $p < 0.05$.

CHAPTER IV

DISCUSSION

The *Drosophila* Toll receptor (Toll-1) and the cytokine Spätzle (Spz1) have been identified as key signaling components of the Toll immune pathway [135], which is not only involved in immune defense by controlling the expression of antimicrobial peptides but also in wound repair responses that promote host tolerance of an infection [205]. The additional Spz homologs (Spz2-6) may encode ligands for other members of the *Drosophila* Toll family, but their role in immunity is less understood [131]. Homologs of *Drosophila* Toll and Spz have been identified in mosquito genomes [82, 206, 207], however, the mosquito Toll pathway remains poorly characterized relative to its *Drosophila* counterpart. For example, in the dengue transmitting mosquito *Aedes aegypti*, three homologs of the *Drosophila* Spz, Spz1A, 1B, and 1C, were identified, yet only *Spz1C* kd by RNAi resulted in increased susceptibility to *B. bassiana* infection. Similarly, out of the 12 Tolls, the *Ae. aegypti,* Toll5A seems so far to be involved in anti-fungal defense through the Rel1 pathway, however it remains unclear whether Spz1C is the ligand for Toll5A [168]. In *Anopheles stephensi*, the fungus *B. bassiana* exports a microRNA-like molecule to suppress host immunity by silencing Spz4, which is highly expressed at the point of fungus penetration, pinpointing a role for Spz4 in providing resistance to fungal infections [208]. In *A. gambiae*, the role of the six Spz genes identified from the published genome sequence remains largely unknown [71]. Hence, we aimed to characterize the Spz gene family at the functional level in this important malaria vector.

None of the Spz genes gave a consistent RNAi phenotype with respect to mosquito survival of infections with the Gram-positive bacteria *S. aureus*, rather mixed phenotypes were observed in the different trials. There could be several explanations for these observations: inefficient silencing of Spz genes by RNAi, functional redundancy between Spz genes, or a minor role for Toll pathway in mosquito tolerance to Gram-positive bacterial infections. Although we observed a reduced expression for all Spz genes after RNAi-mediated silencing, the effect was moderate for several genes specifically for Spz2, Spz4, and Spz5 which exhibited a 37%, 41%, and 51%, reduction in expression, respectively. However, it is difficult to draw definite conclusions in this regard without scoring the Spz protein levels, which is impossible at that stage due to the absence of antibodies against the Spz proteins. Interestingly, Spz2 and Spz4 whose kd significantly compromised survival in more trials compared to the other candidates, seem to be the least efficiently silenced suggesting that a complete knockout of these two genes might trigger more penetrant phenotypes. Although there is no evidence of functional redundancy between Spz genes from other organisms, this point is worth investigating by performing double kds in different combinations; however, without knowing the efficiency of gene silencing at the protein level for the different Spz genes, the results from such studies may be difficult to interpret. Of note, even Rel1, a key transcription factor downstream of the Toll pathway, its kd by RNAi did not seem to compromise mosquito survival to *S. aureus* infections in most trials. In fact, Rel1 kd has given mixed phenotypes in our hands with respect to both tolerance and resistance to *S. aureus*, possibly because of its poor silencing efficiency [39, 170], or because the mosquito Toll pathway is not as efficient and essential as that in *Drosophila* defense against Gram-positive bacteria [112, 113]. This is further supported by the observation

that neither the silencing of Rel1 nor of the six Spz genes compromised the ability of mosquitoes to control *S. aureus* proliferation. Unlike in *Drosophila*, where the Toll pathway plays an essential role in defense against Gram-positive bacteria by controlling the expression of the antimicrobial peptides Bomanins [57, 209], no specific pathways or genes have been convincingly linked to defense against Gram-positive bacteria yet in mosquitoes. We have recently shown that CLIPA7, a modular non-catalytic clip domain serine protease plays an essential role in the clearance of *S. aureus* infections in a melanization-independent manner [210] suggesting that mosquitoes depend on different effector molecules and/or pathways to deal with Gram-positive bacterial infections.

Mosquitoes, like other insects, relay on two forms of innate immune defenses: constitutive and induced. Constitutive defenses are rapidly engaged and peak shortly after an infection (less than an hour), such as phagocytosis and the activation of the melanization response. The activation of AMPs by the Toll pathway is part of the induced response which requires 12 to 48 hours to peak [211], but persists longer. Classically, the quantification of bacteria in whole mosquitoes is done at 24 hours by convenience. However, this point might be considered early if we are to score the full effect of the Toll pathway on bacterial clearance, which might be more accurate at later time points. A study in the beetle *Tenebrio molitor* revealed that the vast majority of bacteria introduced into the hemolymph are cleared quickly by the constitutive defense whereas the induced response characterized by antimicrobial peptide synthesis seems to protect the insect from bacterial persistence in their tissues [212]. Hence, another reason why Spz gene kd in our hands did not influence *S. aureus* clearance may be due to the fact that bacterial proliferation was scored at a relatively early time point. It would be interesting to score the RNAi phenotypes of these genes at several later time points (in

days) after infection to provide a more comprehensive picture of the pattern of bacterial clearance over time.

On the other hand, Spz silencing does not seem to have a significant effect on mosquito resistance to fungal infections, indicating that Toll does not have the main role in fungal clearance, rather TEP1 is the key player [18]. Interestingly, *Spz2* silencing significantly compromised mosquito survival to fungal infections in most of the trials (6 out of 8), whereas the remaining genes gave mixed phenotypes, suggesting that Spz2 may be indispensable for mosquito tolerance to fungal infections. Surprisingly, in these trials, silencing TEP1, a gene known to be required for anti-fungal defense [40] and that is often used as a positive control, did not significantly compromise mosquito survival to *B. bassiana* infections. This could be possibly attributed to the batches of spores used in these assays which might have lost their infectivity if collected from culture plates that were too old.

In contrast to the classical notion established from *Drosophila* studies positing that the Toll and Imd pathways are preferentially activated by Gram-positive bacteria and Gram-negative bacteria, respectively, studies in other insects showed that both pathways are intertwined and not independent [213], and that Toll pathway can be activated by Gram-negative bacteria as well [214], hence the rationale for studying the contribution of mosquito Spz genes in defense against infections with the Gram-negative bacteria *S. marcescens*. The observation that *Spz1-6* kd did not compromise mosquito tolerance to infection with *S. marcescens* suggests that the Toll pathway may not be an essential component of the immune defense against Gram-negative bacterial infections in *A. gambiae*. This may not be surprising due to the key role attributed to the

complement-lectin system composed of TEP1 and the CTL4-CTLMA2 complex in clearing infections with Gram-negative bacteria [94, 96, 215, 216].

Spz gene expression was studied in whole mosquitoes and not in specific tissues where they could be more expressed. For instance, many immune genes are shown to be predominately expressed in insect hemocytes and fat bodies [37, 38, 217]. One study in *Aedes aegypti* focused on Spz genes' expression in specific mosquito tissues, revealing that they are expressed and induced differently: Spz1A was expressed in mosquito fat bodies and ovarian tissues after fungal challenge, whereas Spz1B was only observed in the ovaries and Spz1C mainly in the fat bodies in a non-inducible manner, and none of them were observed in the midgut [168]. Spz expression, which varies in tissue- and time-dependent manners, is shown to be upregulated by bacterial and fungal infections in several organisms [200-204]. For example, in the red palm weevil *Rhynchophorus ferrugineus*, the Spz homolog RfSpätzle was shown to be significantly induced in the fat body after infection with *S. aureus*, *E. coli,* and *B. bassiana* [204]. Consequently, we expected Spz expression to be upregulated in response to microbial infection in *A. gambiae*. We found that Spz expression increased after fungal infection, and peaked at 48 hours post-challenge. Surprisingly, none of the Spz gene expression was induced after infection with *S. aureus*, contradicting previous studies where Spz transcript levels increased after challenge with Gram-positive bacteria in other insect species [200-204], and hinting that the Toll pathway may be more involved in anti-fungal rather than anti-bacterial responses in mosquitoes.

In conclusion, our knowledge of the Toll pathway activation remains largely fragmented in the major malaria vector *A. gambiae*. We found that of the six Spz gene candidates, Spz2 seems to contribute most convincingly to mosquito tolerance to

B. bassiana infections but less to *S. aureus* infections. The fact that most Spz genes were also significantly induced by *B. bassiana* but not by *S. aureus*, suggests that the mosquito Toll pathway may be mainly involved in anti-fungal defenses. The Spz2 RNAi phenotypes obtained in this study warrant further functional characterization of this gene, initially by targeting its transcript with a combination of dsRNAs to induce a better level of silencing. Functional and molecular characterization of the key components of the immune response in *A. gambiae* is expected to advance our knowledge of host-pathogen interactions in this important disease vector. The characterization of the Toll pathway could be particularly attractive since it seems to be essential in anti-fungal defense and fungi are being considered as promising biopesticides for the control of mosquito vectors of diseases [189-191]. Such knowledge would lead to a better understanding of fungal evasion mechanisms of mosquito immune responses and help better evaluate the success of future fungal biopesticides of mosquitoes in the field.

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